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USE OF RECOMBINANT DNA TECHNIQUES FOR THE PRODUCTION OF A MORE EFFECTIVE ANTHRAX VACCINE

ANNUAL REPORT

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We report an improved isolation proceudre for the preparation of pX01 and pX02 plasmids of B. anthracis. These plasmids have been physically characterized with regard to bouyant density, GC content and size analysis using restriction enzyme digestions. Restriction maps of these DNAs have been generated. pX01 is 175 kbp and pX02 is 95 kbp. The location of the toxin genes, protective antigen (PA), lethal factor (LF) and edema factor (EF), have been positioned on pX01.

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19. The EF gene has been cloned and sequenced. Unique features of the EF gene include a putative ATP binding site, a 33 amino acid leader sequence and a very good ribosome binding site. The direction and location for the start of each of the toxin genes have also been determined. EF has been expressed in enzymatically active form and is full-length. Expression vectors in E. coli have been used to produce large quantities of this protein. Expression vectors for LF and PA in both E. and coli and B. subtilis have been used for enhanced expression of these genes as well.

In order to generate a safe anthrax vaccine using recombinant DNA techniques, we have begun experiments to specifically mutant each of these toxin genes to generate non-functional proteins that retain most, if not all, of their immunogenic properties. For example, the trypsin cleavage site of PA is being altered to remove the Arg-Lys-Lys-Arg sequence which is cleaved to activate PA. In addition, the putative ATP binding site of EF is being mutated to prevent enzyme activity. These experiments should generate mutant toxin proteins which will be safe vaccine components which will then be tested in animals.

SUMMARY OF RESEARCH

The overall goal of the present research is to construct a safe, effective human anthrax vaccine using recombinant DNA techniques. These studies are broken down into three phases:

Phase I. Isolation and characterization of the Bacillus anthracis toxin genes for protective antigen (PA), lethal factor (LF) and edema factor (EF). The individual toxin genes will be cloned in expression vectors for large scale production of toxin proteins using E. coli and B. subtilis. These experiments should provide enhanced production of the different toxin components which are made in low levels in E. coli.

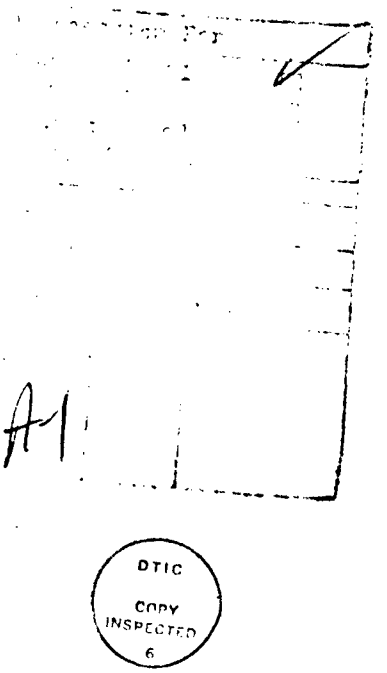
Phase II. Generation of mutant toxin proteins from cloned toxin genes defined in Phase I. Mutations derived from deletion analysis or site-specific mutagenesis of the cloned toxin genes will be generated using in vitro manipulations of the recombinant plasmid DNAs. Mutations of potential use for vaccine construction will be identified as those which are non-toxic but still immunologically active and protective.

Phase III. Insertion of mutant genes back into B. anthracis with the selective removal of wild-type genes. Then, testing of these mutant strains will be performed in animals, such as the mouse or guinea pig.

The research outlined in this annual report describes the cloning and characterization of the individual B. anthracis toxin genes. These genes are being expressed in B. subtilis and E. coli and are being specifically mutated to generate mutant derivatives which lack biochemical activity but maintain immunological properties. In addition, a physical characterization of the B. anthracis plasmids with regard to size, genetic complexity, GC% and restriction enzyme mapping is also described.

FOREWORD

The investigators (Principal Investigator and Graduate Students) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (May 1986). Supplemental guidelines pertaining to the subcloning of the individual B. anthracis toxin genes in sporulation competent B. subtilis was approved by the NIH committee on toxins March 13, 1986.



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BACKGROUND

As discussed in the summary, the goal of the experiments performed in this laboratory is to develop a more effective human anthrax vaccine for the protection of U.S. Army troops using recombinant DNA techniques. The current human anthrax vaccine consists of alum-precipitated supernatant material from fermenter cultures of B. anthracis which consists predominantly of PA (protective antigen) (9). Unfortunately, this vaccine may not be effective against all strains of B. anthracis since several virulent strains have been classified as "vaccine resistant" with regard to this human vaccine (19). Clearly, an effective vaccine must afford immunological protection against all strains of B. anthracis and against all forms of infection, including aerosol.

Virulent strains of B. anthracis contain two different plasmids. The toxin plasmid (pXO1) is necessary for expression of the three toxin proteins (5,13) and the capsule plasmid (pXO2) is necessary for production of the poly-D-glutamic acid capsule (2,16). In order to be able to insert mutant toxin genes back into B. anthracis for the production of a safe vaccine strain it has been necessary to characterize these plasmids. Studies designed to physically characterize these plasmid are now essentially complete. These analyses have included bouyant density centrifugation, DNA melting analysis and restriction endonuclease mapping of these DNAs. These characterizations should be helpful in generating recombinant vaccine strains of B. anthracis.

Each of the anthrax toxin genes are now cloned. The PA and EF genes have been sequenced. Experiments which are aimed at expressing these toxin genes in large quantities in E. coli and B. subtilis are in progress. In addition, we are specifically mutating the different toxin genes in

order to generate mutant toxin proteins which are still immunogenic but biochemically non-functional to be used in the vaccine development.

MATERIALS AND METHODS

The procedures described here pertain to the isolation and characterization of B. anthracis plasmids pXO1 and pXO2 and to the cloning and analysis of the PA, LF and EF genes. They are included to provide information prior to publication for the benefit of other investigators.

Isolation of pXO1 and pXO2. Our plasmid isolation procedure is a modification of the protocol described by Green et al. (2). A single colony of the Sterne (25) or Pasteur (ATCC 6602) strain of B. anthracis was incubated at 37°C in a shaking incubator for 14 to 16 hr in one liter Penassay broth (Difco) or L-broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter [pH 7.5]). The cells are harvested by centrifugation at 8,000 rpm (Sorvall GSA rotor) and the pellet resuspended in 50 ml E-buffer (0.04 M Tris-acetate [pH 7.9], 2 mM EDTA). Cells are lysed by the addition of 100 ml of freshly-made lysis solution (50 mM Tris Base, 15% w/v sucrose, 3% sodium dodecyl sulfate [SDS], 0.5 M NaOH) and incubated with occasional stirring at 55-60°C for 30 min. After centrifugation at 10,000 rpm (GSA rotor) for 30-60 min to pellet cellular debris and unlysed cells, the plasmid-containing supernatant was extracted once or twice with an equal volume of unbuffered phenol/choroform (1:1). The aqueous phase was then incubated on ice for 5-10 min and neutralized with 25 ml ice-cold 2 M Tris-HCl [pH 7.0]. The solution was then adjusted to 0.3 M NaOAc [pH 5.4] and ethanol precipitated using two volumes of 95% ethanol. Following a 30 min incubation at -20°C, the nucleic acid was collected by centrifugation at 10,000 rpm. The pellet was dissolved in 10 ml of RNase buffer (100 mM Tris-HCl [pH 7.4], 10 mM EDTA) containing 100 μg/ml RNase A and incubated at 37°C for 30 min. This solution was adjusted to 1% SDS and 50 μg/ml proteinase K and incubated at 65°C for 20 min. After extraction

with equilibrated phenol/chloroform (aqueous phase contained 50 mM Tris-HCl [pH 8.6], 0.1 M NaCl, 2.5 mM EDTA, 1% 2-mercaptoethanol) and ethanol precipitated, the DNA pellet was dissolved in TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA) buffer and adjusted to 0.5 M NaCl. This semi-pure plasmid was then chromatographed on a NACS-37 reverse phase ion-exchange column (Bethesda Research Labs) to remove genomic DNA. A gradient of 0.50-0.80 M NaCl (in TE buffer) was used to elute the supercoiled plasmid. After ethanol precipitation and a wash with 95% ethanol, the plasmid was ready for further analysis.

Generation of EF recombinant DNA library. Toxin plasmid pXO1 was partially cleaved with MboI and then ligated into BamHI cleaved pUC8 as described previously (10). Recombinant E. coli from this DNA library were grown as colonies on grided L-agar plates and then transferred to nitrocellulose filters, lysed and screened immunologically (10). Nitrocellulose filters of identically grided plates were also prepared for hybridization and processed as described by Maniatis et al. (17). They were hybridized to a synthetic oligonucleotide which had been labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The filters were hybridized overnight at 30°C with the radiolabeled oligonucleotide in 6X SSPE, 5X Denhardt's solution and 0.5% SDS. After hybridization the filters were washed three times for 15 min in 6X SSPE, 0.5% SDS at 30°C and autoradiographed overnight at -70°C with Kodak XAR-5 film. Following autoradiography plasmids pEF68, pEF194 and pEF215 were isolated which hybridized to the EF oligonucleotide. An additional recombinant plasmid, designated pSE42, which contained a single BamHI fragment cloned into the BamHI site of pBR322 (13) also hybridized to this oligonucleotide.

Determination of EF, PA and LF gene orientations. In order to determine

gene orientation and the start of the different toxin genes, we have generated several recombinant plasmids which contained the appropriate DNAs which hybridized to the amino terminus-specific oligonucleotides for each gene. The 2.1 kbp HindIII fragment from pPA26 (13), the 2.2 kbp PstI fragment from pLF7 (10) and the 2.2 kbp HindIII fragment from pSE42 (see Figure 5) were each cloned into pTZ18R (11). Following the preparation of single-stranded plasmid (11) for each of these recombinant plasmids, radiolabeled oligonucleotides specific for the PA, LF or EF genes were hybridized to the appropriate DNAs. Based on which strand hybridized to a specific oligonucleotide, the direction of toxin gene transcription was determined. Then, using the same radiolabeled oligonucleotides we synthesized DNA using the Klenow fragment of E. coli DNA polymerase I. After a 60 min incubation at 37°C, this polymerization mixture was ethanol precipitated, dissolved in restriction enzyme buffer and digested with different restriction endonucleases. These DNA mixtures were then electrophoresed on a 4% denaturing polyacrylamide gel (53). Following autoradiography, the size of the resultant radiolabeled DNAs (from the start of the primer to the enzyme cleavage site) was determined. Based on the known restriction maps of these cloned DNAs, we could precisely position the start of each toxin gene.

RESULTS

pXO1 and pXO2 plasmid isolation. The plasmid isolation procedure described here is a variation of the protocol described by Green et al. (2), but includes NACS-37 chromatography to isolate supercoiled DNA. This chromatography step efficiently separates small amounts of genomic DNA from plasmid. The avirulent B. anthracis Sterne strain (25), which produces toxin but no capsule, contains pXO1 and the Pasteur strain (ATCC 6602), which is capsule positive but toxin negative, contains pXO2 (2). The different steps in plasmid isolation are listed in the legend of Figure 1 and are essentially identical for pXO1 and pXO2. Since B. anthracis cells are almost completely refractile to lysozyme digestion, we employed a heat-alkaline treatment to lyse the cells (2,21) as described in Methods. Supercoiled plasmid is clearly present in these preparations (see Figure 1). Lane 3 shows the DNA in its final state of purification, but without NACS chromatography. As the plasmid isolation proceeded, a shift in the proportion of supercoiled DNA (upper band) to the relaxed or linearized plasmid (lower band) often occured. This conversion was also observed after prolonged storage of supercoiled DNA, and manipulations which tended to shear the DNA, such as freeze-thaw cycles and repeated phenol/chloroform extractions, converted supercoiled DNA to the relaxed or linearized form. As a result, the originally isolated supercoiled DNA often migrated in the relaxed region of the agarose gels following storage (data not shown). The region of the gel which contained relaxed pXO1 and pXO2 is the same region other investigators (2,5,22) suggested that genomic DNA migrated. It should be noted that these large plasmids are easily sheared, converting them from supercoiled to relaxed or linear DNA, even banding them in CsCl gradients converts supercoiled to relaxed

DNA. Therefore, since genomic DNA bands at the same position as the relaxed plasmid (unpublished observation of the author), CsCl gradients cannot be used to effectively separate genomic DNA from the relaxed plasmid. A typical yield of pXO1 from a one liter culture of B. anthracis was about 200 μg, which is close to the maximum amount of DNA expected per liter of culture if these plasmids were present as single copies within B. anthracis cells.

The plasmid DNA shown in Figure 1, lane 8, can be used for restriction endonuclease analysis (see lane 9), but in order to remove any remaining genomic DNA we used NACS-37 reverse-phase ion exchange chromatography to isolate pure plasmid. Supercoiled DNA eluted at 0.69 M NaCl, which is characteristic of supercoiled DNA using NACS resins. Figure 2A shows pXO1 DNA that was purified using NACS chromatography and then digested with different restriction endonucleases. The lack of background flourescense indicated that genomic DNA was removed. Figure 2B shows purified pXO2 DNA, but without NACS chromatography. Some contaminating genomic DNA is clearly still visible. Tables I and II show the sizes of restriction enzyme cleaved pXO1 and pXO2, respectively. Total size of pXO1 is about 175 kbp and pXO2 is 95 kbp. When these DNAs are cleaved with EcoRI, which cleaves many times, several DNA bands are doublets and must be counted more than once to give the correct plasmid size.

Plasmid DNA melting curves. For T_m analysis, the NACS-purified plasmid DNAs were sonicated in TE buffer to a size of approximately one kbp. The absorbance at 258 nm was monitored while the temperature was varied from 60-100°C in 0.5°C increments (Figures 4). The sharp hyperchromic shift for both plasmid DNAs indicated that denaturation was taking place. The T_m's (the average of at least 6 experiments) for pXO1 and pXO2 was 82.5°C

± 0.3°C and 82.2°C ± 0.3°C, respectively. These DNA melting points correspond to GC contents of 32.2% for pXO1 and 31.5% for pXO2.

Bouyant density determinations. For CsCl banding, approximately 0.5 μg of plasmid DNA was radiolabeled using [α-^{32}P]dCTP by nick translation. Following purification, the radiolabeled DNAs were added to 3.5 ml of a 53.0% CsCl solution in TE buffer. After an isopycnic gradient was generated the DNA samples were fractionated into 0.15 ml aliquots and the amount of radioactivity in each sample was determined using liquid scintillation. The density of each fraction was determined from the refractive index at 20°C. Analysis of several different banding profiles showed that pXO1 and pXO2 had average densities of 1.690_5 and 1.690_8, respectively. These values corresponded to GC contents of 31.1% for pXO1 and 31.4% for pXO2. These values are close to the GC% of B. anthracis genomic DNA which is 32.2%.

Restriction maps of pXO1 and pXO2. The restriction maps for pXO1 and pXO2 are essentially completed. Figure 3 shows the current map for pXO2. Construction of this restriction map used the Southern Cross mapping procedure recently developed by Potter and Dressler (20). As shown in Figure 3, we have a restriction map for the enzymes PstI, SstI, BamHI and ClaI as well as for BglII and PvuII (not shown) but not yet for EcoRI and HindIII, which cleave pXO2 at least 20 times each. Using recombinant plasmids of an EcoRI digest, we should be able generate a restriction map for these two enzymes as well.

The restriction map for pXO1 (Figure 4) is essentially finished for the enzymes PstI, SstI, BamHI, PvuII, ClaI and SalI. However, due to the size of pXO1, in comparison to pXO2, we generated a restriction map using recombinant λ phage DNAs and BamHI/SstI cleaved pXO1 DNA isolated from agarose gels. There are still two areas of some ambiquity, but these are

in the process of being refined. The map shown in Figure 4 is essentially complete with little or no variation for the enzymes shown (PstI, SstI, BamHI). The positions of the LF, PA and EF genes are also shown. We do not have a λ recombinant which contains both LF and PA genes. These restriction maps should be beneficial in examining variations between different B. anthracis strains, and for genetic modifications, such as for transposon mutagenesis, for construction of mutant toxin derivatives and for DNA sequencing studies.

Cloning of edema factor gene. The edema factor is a calmodulin-dependent adenylate cyclase that has been well characterized (3,4). Although previous attempts to clone the EF gene were unsuccessful, we have now cloned and expressed this gene in E. coli. The EF gene was cloned as a 6.5 kbp BamHI fragment in pBR322. This plasmid, designated pSE42, along with other recombinant plasmids isolated from a pXO1 library, was positively identified as containing at least part of the EF gene by hybridizing with a radioactively labeled EF-specific oligonucleotide. The sequence of this oligonucleotide (5'-ATGAAPGAXCAPTAP-3'; where X=G,A and P= T,C) was deduced from the amino acid sequence for the first 5 amino acids of EF (Met-Asn-Glu-His-Tyr; determined by Dr. J. Schmidt [USAMRIID]). A restriction map of pSE42 is shown in Figure 5.

The 2.2 kbp HindIII fragment from pSE42 (designated as EF2.2 in Figure 5), which hybridized to the labeled oligonucleotide (data not shown) was cloned into plasmid pTZ18R (36) in both orientations. pTZ18R contains the IG region from M13 and can be used to produce single-stranded DNA for strand-specific hybridization. One of these recombinant plasmids, designated pEF2.2-11, hybridized with the radiolabeled oligonucleotide. Based on the orientation of the 2.2 kbp HindIII fragment in this plasmid, the

deduced direction of transcription of the EF gene was in a counter clockwise direction as depicted in Figure 5.

In order to precisely position the start of the EF gene, we used the labeled EF oligonucleotide for primer elongation. Following polymerization with the Klenow fragment of DNA polymerase I, the reaction mixtures were cleaved with HindIII. The size of the resultant DNA from the start of the labeled primer to the HindIII cleavage site was about 800 bp, indicating that the amino terminus of EF is about 800 bp to the right of the HindIII recognition site with transcription proceeding in the leftward direction. Cleavage of the same elongation product with XbaI generated a fragment of about 450 bp, consistent with the map positions of XbaI and HindIII. Clearly, pSE42 contains enough DNA to encode the entire EF gene.

pSE42 was sent to Dr. Francine McCutchen of Meloy Laboratories for DNA sequencing. The entire EF gene sequence has now been determined and is shown in Appendix I. Several interesting features of the EF sequence are present. (i) EF apparently contains a 33 amino acid leader sequence (beginning at nucleotide 554 to 643 on the DNA sequence) which is probably necessary for secretion. A similar 29 amino acid leader sequence was also found for PA (LTC. J. Lowe and Dr. S. Welkos, personal communications). (ii) A very strong ribosome binding site immediately upstream from the start of the EF protein is present (between nucleotides 528 and 537). (iii) Amino acid residues 314 to 321 (corresponding to nucleotides 1582 to 1605 of the DNA sequence) of the mature protein contain the sequence Gly-X-X-X-X-Gly-Lys-Ser which is a perfect match to a consensus sequence which was recently reported to be present in prokaryotic and eukaryotic ATP binding proteins (52) and which is part of their ATP binding sites. It is probable that this sequence is part of the EF ATP binding site as

well. (iv) No homology between the EF gene sequence or the deduced EF protein sequence was observed with the reported nucleotide and protein sequences from GenBank, including yeast and E. coli adenylate cyclases. (v) There appears to be no good consensus promoter immediately upstream from the start of the EF sequence, although a DNA sequence located at positions 317-348 seems to resemble a B. subtilis promoter, albeit a poor one. It should be noted that the PA promoter, which immediately precedes the PA gene, is a perfect match to a consensus B. subtilis promoter (data not shown).

Position for the start of the LF and PA genes. Using radiolabeled oligonucleotides specific for the first five amino acids of PA and LF, we have determined the position for the start of these genes as well. When the PA oligonucleotide, which corresponds to the first 5 amino acids of mature PA (Glu-Val-Lys-Gln-Glu), was hybridized and elongated using Klenow DNA polymerase and then digested with HindIII, a DNA about 300 bases was generated. After these experiments were performed, the PA sequence was determined and the actual distance between the position of the PA oligonucleotide and the HindIII site is 295 bases. Likewise, when the LF oligonucleotide, which corresponds to the beginning of LF (10) was used in these experiments, a DNA about 800 bases was generated when cleaved with EcoRI. Consequently, the start of the LF protein occurs about 800 bases to the right of the EcoRI cleavage site located to the left of the ClaI site in pLF7 (see Figure 2 in reference 10).

Expression of EF in E. coli. We have not been able to detect expression of EF in E. coli carrying pSE42, even though it contains the entire EF gene. Therefore, we attempted to express the EF gene by fusing it to the lac promoter. The 4 kbp EcoRI to BamHI fragment from pSE42 (see Figure

5) was subcloned into pTZ18R. This construction, designated pEF42, positioned the EF gene about 400 bases downstream from the lacZ promoter, but in the same transcriptional orientation. When this plasmid was grown in E. coli, EF was produced. IPTG, an inducer of the lac promoter, increased EF production at least 2-fold. pTZ18R also contains the T7 RNA polymerase promoter downstream from the lac promoter. Using an E. coli strain provided by Dr. W. Studier (Brookhaven National Laboratories) which contains a copy of T7 RNA polymerase under the contol of the lac promoter (14), we monitored expression of EF after induction of T7 RNA polymerase by IPTG. In the presence of IPTG, we detected enzymatically active EF and full-length EF (89,000 Da) was observed in Western blots (S. Leppla, personal communication). We have also fused the EF gene, using site-specific mutagenesis, directly to the lac promoter and detected large quantities of full-length protein and enzyme activity.

Expression of elevated levels of B. anthracis toxin protein in E. coli and B. subtilis. Each of the B. anthracis toxin genes, PA (13), LF (10), and EF (manuscript in preparation), have been cloned and expressed in E. coli. We are now attempting to enhance expression in E. coli and in B. subtilis. For example, using the T7 RNA polymerase expression system developed by Dr. W. Studier (14), we have successfully expressed high levels of enzymatically active EF (3,4). Drs. B. Ivins and S. Welkos successfully transferred the PA gene into B. subtilis and monitored the high level of expression of PA (18). We are also generating genetic constructions which will fuse the EF gene to the PA promoter. Since the PA promoter is very active in B. subtilis (18), we should be able to produce large quantities of EF, perhaps in similar quantities to the level of PA.

In order to be able to shuttle mutant toxin genes from E. coli to B. subtilis, it will be necessary to use plasmids which replicate in both organisms (6-8,12). This conventional approach so far has been only marginally successful. However, we have transferred the PA, LF and EF genes into B. subtilis using a slightly different approach. Normal and mutant toxin plasmids are grown initially in E. coli, then, as the last step of mutagenesis, a B. subtilis plasmid is ligated to the toxin plasmid. For example, using E. coli plasmids (11) which contain the toxin genes, we have combined them with separate B. subtilis plasmids (e.g., pC194, pE194, pUB110, pBS42 [1] or pGX284 [23,24]). The resultant chimeric plasmids which contain toxin DNA, E. coli plasmid DNA and B. subtilis plasmid DNA are then transformed into B. subtilis. The advantage of using this experimental approach is that the toxin genes are easily manipulated and modified in E. coli and then transfered into B. subtilis using efficient protoplast or competent cell transformation. These chimeric plasmids are stable in E. coli and transform B. subtilis well.

Construction of mutant PA and EF genes. We have initiated experiments that should be result in the construction of mutant toxin genes. If biochemically non-functional toxin mutants are generated which are still immunogenic, these proteins can be used safely as vaccine components. Dr. S. Leppla and Col. A. Friedlander have identified a site in PA which is cleaved by a trypsin-like enzyme when bound to a receptor on the cell surface (personal communications). This proteolytic cleavage of PA is required for the binding of LF or EF before endocytosis. The amino acid sequence at this location in PA is Arg-Lys-Lys-Arg. We have constructed, using site-specific mutagenesis, a mutant derivative of the PA gene in which we have inserted SstI and SalI sites which flank this cleavage

site. The intent of this alteration is to remove and to alter the basic amino acids (Arg-Lys-Lys-Arg) at this location and then to substitute other amino acids. These substitutions should prevent proteolytic cleavage and render PA non-functional but still fully immunogenic.

As described above, an amino acid sequence in EF (from amino acid residues 314 to 321 of the mature protein) which is Gly-X-X-X-X-Gly-Lys-Ser is a perfect match to a consensus sequence present in prokaryotic and eukaryotic ATP binding proteins (15). Since the Lys residue is part of the ATP binding site it is likely that this Lys residue is also part of the EF ATP binding site. Since the overall goal of this research contract is to generate a safe anthrax vaccine, this conserved amino acid sequence is a good candidate for site-directed mutagenesis. If this Lys residue were replaced with a non-charged amino acid, it is possible that EF will no longer bind ATP and that EF will be enzymatically inactive. These mutagenesis experiments are in progress.

CONCLUSIONS

It appears from the data reported here that Phase I and Phase II of the original proposal are nearing completion. Each of the anthrax toxin genes have been cloned and expressed in E. coli and to some extent in B. subtilis. Current research and experiments to be carried out during the first part of this next year should result in the elevated expression of mutant and wild-type toxin protein. With the research at the current level, we should be able to start experiments outlined in Phase III during this year. Therefore, during the next year, we will continue to express the toxin proteins at high levels and will test these proteins for biochemical activity.

We have also succeeded in physically characterizing the toxin plasmid

(pXO1) and the capsule plasmid (pXO2) of B. anthracis. These analyses should be extremely beneficial in the construction of mutant vaccine strains and for DNA sequencing analysis of these plasmids.

PUBLICATIONS

The following articles were published during this reporting period:

Robertson, D. L., and S. H. Leppla. 1986. Molecular cloning and expression on E. coli of the lethal factor gene of B. anthracis. Gene 44:71-78.

The following manuscripts were submitted for publication:

Kaspar, R. L. and D. L. Robertson. 1987. Purification and analysis of Bacillus anthracis plasmids pXO1 and pXO2. submitted to J. Bacteriol.

Tippetts, M. T. and D. L. Robertson. 1987. Molecular cloning of the edema factor gene of Bacillus anthracis. submitted to Gene.

The following dessertations were published:

Tippetts, M. T. 1986. Molecular cloning of the chloroplast genome of Carthamus tinctorius L. and of the edema factor gene from Bacillus anthracis. Department of Chemistry, Brigham Young University.

Kaspar, R. L. 1986. Purification and characterization of pXO1 and pXO2 plasmids from Bacillus anthracis. Department of Chemistry, Brigham Young University.

The following abstracts were published during this reporting period.

Kaspar, R. L. and D. L. Robertson. Purification and analysis of Bacillus anthracis plasmids pXO1 and pXO2. Abstr. Annu. Meet. Am. Soc. Microbiol. 1987.

Tippetts, M. T., D. L. Robertson and R. Leavitt. Molecular cloning and characterization of the Bacillus anthracis edema factor gene. Abstr. Annu. Meet. Am. Soc. Microbiol. 1987.

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TABLE I. Computer-assisted sizing analysis of pXO1 DNA cleaved with different restriction enzymes[a].

BamHI + SalI		PstI		ClaI	
Measured Mobility (cm)	Measured Size (kb)	Measured Mobility (cm)	Measured Size (kb)	Measured Mobility (cm)	Measured Size (kb)
1.53	25.4	1.79	20.7	1.81	20.4
1.62	23.6	1.91	19.0	2.06	17.1
1.65	23.1	2.02	17.6	2.24	15.2
1.86	19.7	2.54	12.6	2.61	12.1
2.26	15.0	2.73	11.3	2.69	11.6
2.30	14.6	2.73	11.3	2.77	11.1
3.55	7.6	2.83	10.7	2.80	10.9
3.71	7.1	3.01	9.8	3.24	8.7
3.75	7.0	3.20	8.9	3.79	6.9
3.94	6.5	3.96	6.4	3.83	6.8
4.02	6.3	3.98	6.4	4.00	6.3
4.23	5.8	4.12	6.1	4.33	5.6
4.33	5.6	4.26	5.8	4.42	5.5
5.73	3.7	4.45	5.4	4.61	5.2
		4.63	5.1	4.68	5.0
Total	171.1	4.76	4.9	4.82	4.8
		5.01	4.6	4.97	4.6
		5.53	4.0	4.98	4.6
		6.09	3.4	5.08	4.5
				5.17	4.4
		Total	174.1	5.51	4.0
				Total	175.3

[a]pXO1 DNA was cleaved with the indicated enzymes, electrophoresed and the ethidium bromide-stained bands photographed. The extrapolated sizes are shown along with the distances migrated in the agarose gel. DNA bands that appeared more intense than expected we counted as doublets. (The mobility distances shown in this Table are those of the Polaroid negative of the ethidium bromide stained gel and are about 40% of the actual migration distances.) λ DNA, cleaved with HindIII, was used as molecular weight standards to establish the sizing curve.

TABLE II. Computer-assisted sizing analysis of pXO2 cleaved with ClaI[a].

Measured Mobility (cm)	Calculated Size (kb)
1.330	24.8
1.540	19.7
1.800	15.5
2.130	11.3
3.040	6.2
3.420	5.2
3.840	4.4
3.870	4.4
5.340	2.6
5.640	1.2
Total	95.3

[a]pXO2 was cleaved with ClaI and then processed exactly as described in TABLE I.

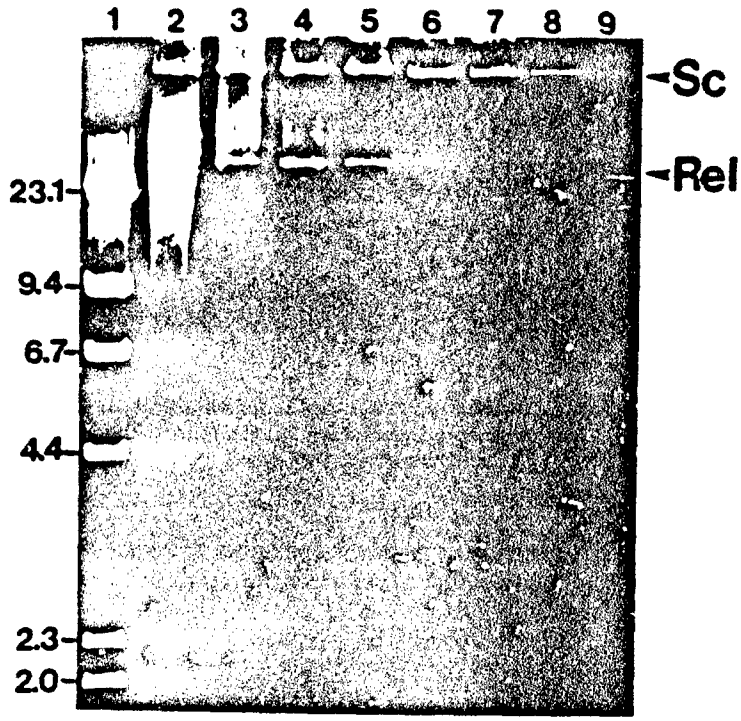

Figure 1. pXO1 DNA following each of the different steps in the plasmid isolation. Lane 1, HindIII digested λ DNA. Lane 2, pXO1 after the heat-alkali treatment and centrifugation. Lane 3, plasmid DNA after neutralization with 2 M Tris-HCl [pH 7.0]. Lane 4, DNA after the first unbuffered phenol/chloroform extraction. Lane 5, pXO1 DNA following second unbuffered phenol/chloroform extraction. Lane 6, plasmid DNA dissolved in RNase buffer. Lane 7, plasmid DNA following RNase A and proteinase K treatment and extraction with buffered phenol/chloroform. Lane 8, pXO1 DNA dissolved in TE buffer. Lane 9, pXO1 DNA (same DNA shown in lane 8) digested with 10 units of BamHI. (Sc-Supercoiled plasmid DNA. Rel-Relaxed or linearized plasmid DNA.)

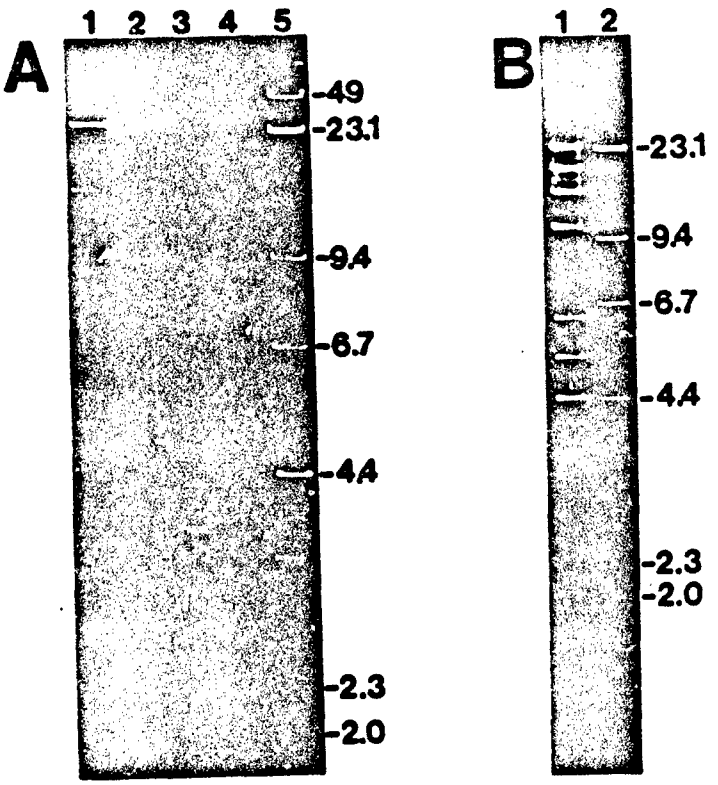

Figure 2. Analysis of plasmid DNAs with various restriction endonucleases. (A) pXO1 DNA (purified over NACS-37 ion-exchange column) digested with BamHI and SalI (lane 1), BamHI and SstI (lane 2), PstI (lane 3), and ClaI (lane 4). Lane 5 is HindIII cleaved λ DNA with fragment sizes shown in kilobases. (B) pXO2 DNA (not purified with NACS chromatography) digested with ClaI (lane 1). Lane 2 is HindIII cleaved λ DNA. A more complete restriction enzyme analysis of pXO1 and pXO2 will be published elsewhere (DLR, S. Simpson and T. Bragg, personal communication).

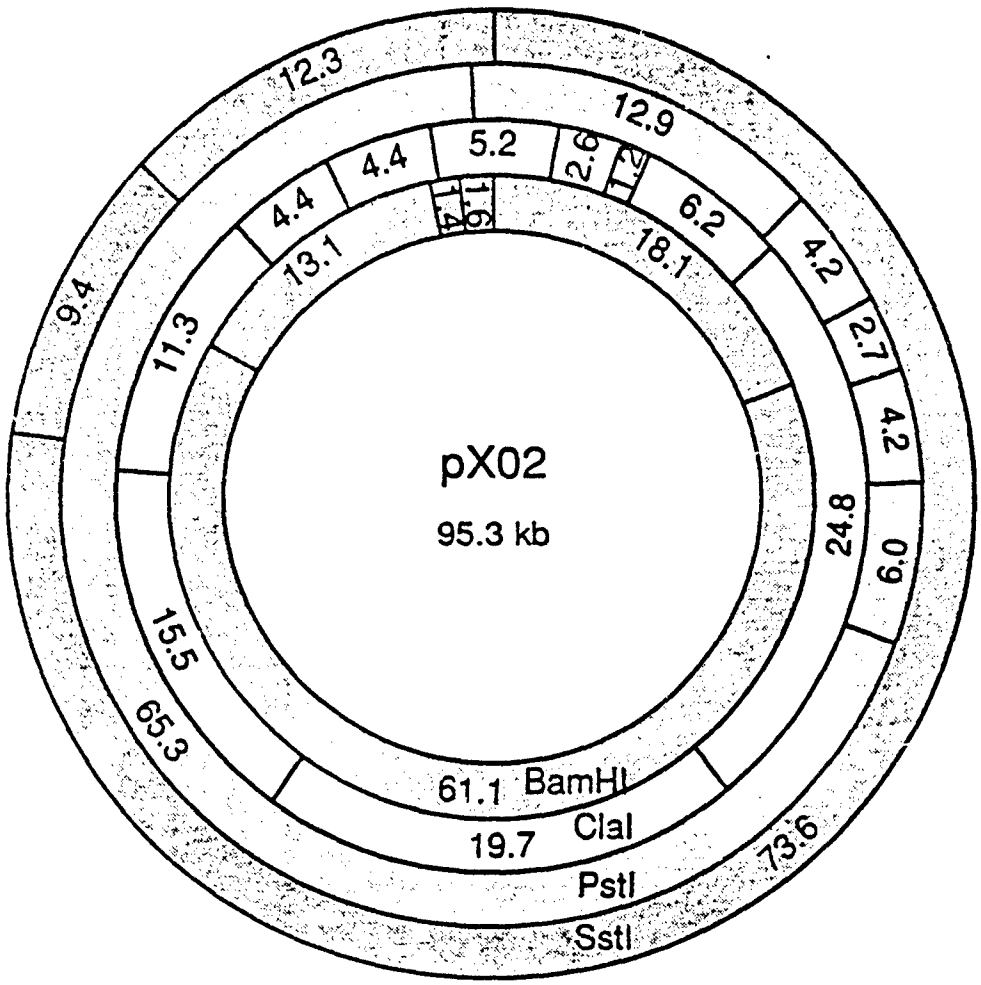

Figure 3. Restriction map of pX02.

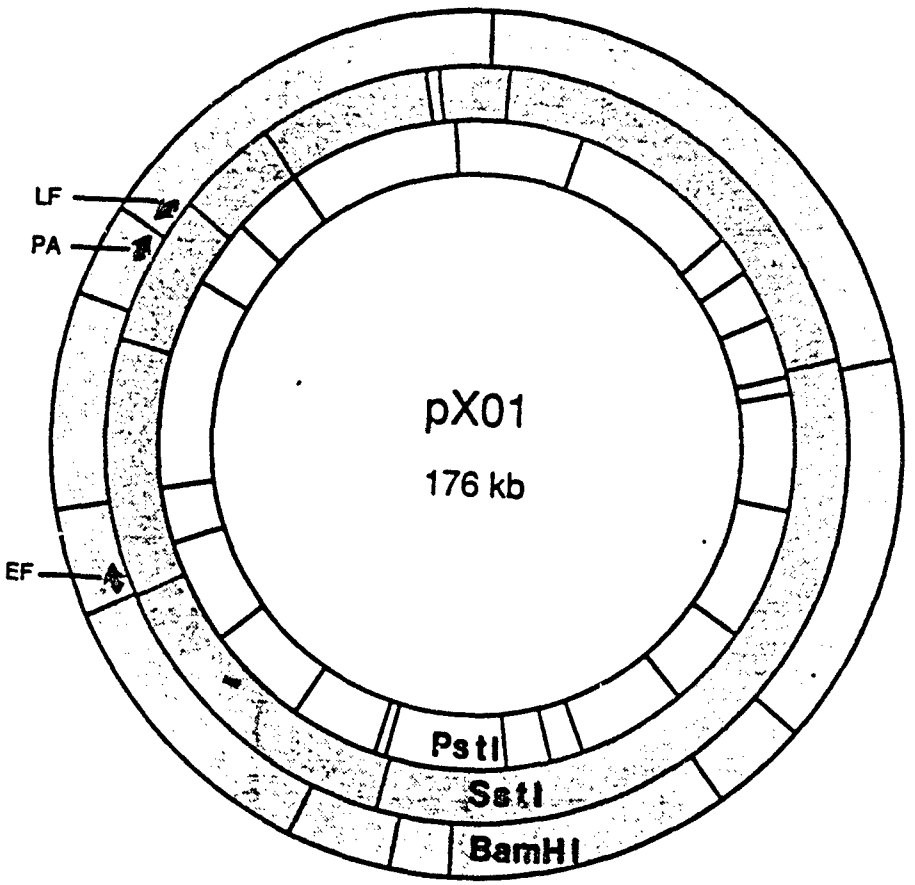

Figure 4. Restriction map of pX01. The positions of the LF, PA and EF genes are depicted. The sizes of DNA fragments for each enzyme are not included due to the lack of space.

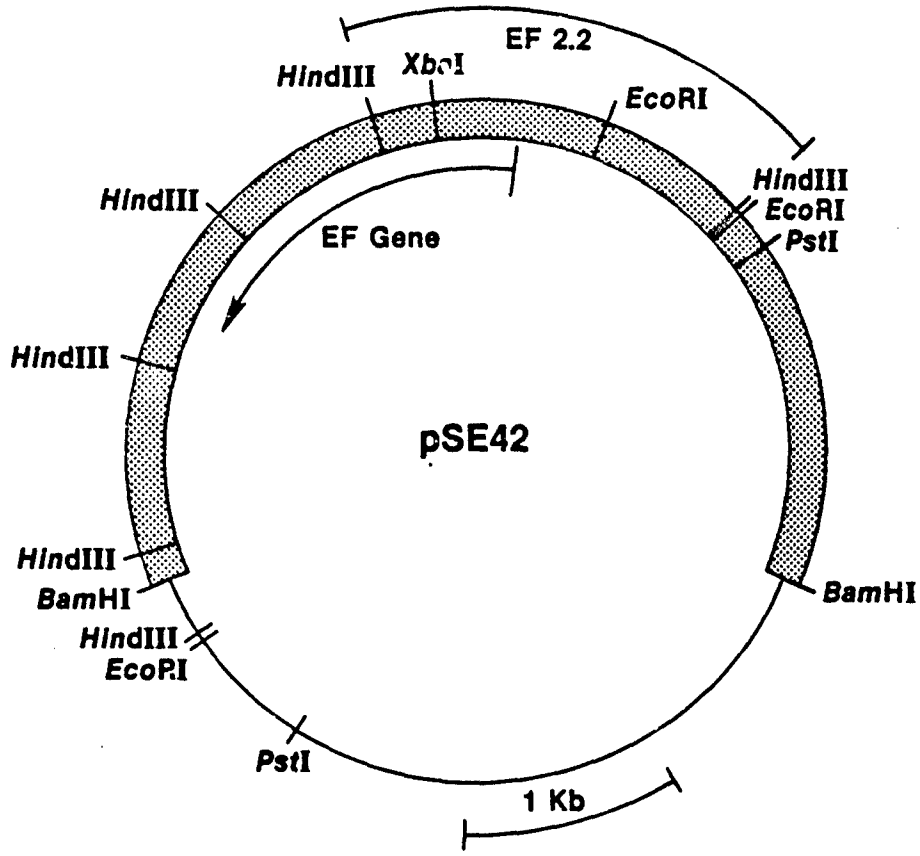

Figure 5. Restriction map of pSE42. The thin line represents pBR322 sequences and the wide line is from pX01 which contains the entire EF gene. The position for the start of EF is shown and the 2.2 HindIII fragment (designated EF2.2) which hybridizes with the EF oligonucleotide and contains the EF promoter.

APPENDIX I. DNA and protein sequence of EF

DNA and protein sequence of EF

```
        10         20         30         40         50         60
TTACTTTTTTATATACTGAATTAAAAAGTCCAAGCACTTATATCGTAATAGATGCTTTCT

        70         80         90        100        110        120
ATTGACCTTATAGTCCTTGAAGTTACGACTGACCAATTATGAGACGTTTGCGCTAACCTG

       130        140        150        160        170        180
CTGAATTCAAAATCGGACTTAGAAATACACATATAGAAATAAACAACCTAATCCATGTCA

       190        200        210        220        230        240
CTGTACCGTTTTTTTACTAAATAAACGAAATCAGTGTAAAAATGAACAGCTGAACTTTAT

       250        260        270        280        290        300
CAACTTAGAATCTCTTTTTTTACTTTAAATGCCTAGCTGTTTTTTCTAATGTTTGTATTT

       310        320        330        340        350        360
CTAAATATATTTAAATATGAATTGTAGCTGTGTGCCAAGAGTTATAATTAATTTAAATAA
                -35 (putative promoter site) -10

       370        380        390        400        410        420
GATTATATTTGTAAATAAAATTGTAATTTAACATGTAGAATAAAGAGATTTTTAGTTTTA

       430        440        450        460        470        480
TTAACAGGATGAAAATCCATAAAACCGTAAATGTGATTTCTAAATTAGTTTAAAATAAAA

       490        500        510        520        530        540
AACAAGGATTTGCTCAGACTTGAGATGAATATCTAAATATCAAGAACCAAAGGAGGTTTA
                                              ribosome binding site

  +1   550        560        570        580        590        600
AGAATGACTAGAAATAAATTTATACCTAATAAGTTTAGTATTATATCCTTTTCAGTATTA
  MetThrArgAsnLysPheIleProAsnLysPheSerIleIleSerPheSerValLeu
                    33 amino acid leader sequence

       610        620        630        640        650        660
CTATTTGCTATATCCTCCTCACAGGCTATAGAAGTAAATGCTATGAATGAACATTACACT
LeuPheAlaIleSerSerSerGlnAlaIleGluValAsnAlaMETAsnGluHisTyrThr
                                          1st amino acid of EF

       670        680        690        700        710        720
GAGAGTGATATTAAAAGAAACCATAAAACTGAAAAAAATAAAACTGAAAAAGAAAAATTT
GluSerAspIleLysArgAsnHisLysThrGluLysAsnLysThrGluLysGluLysPhe
```

```
      730       740       750       760       770       780
AAAGACAGTATTAATAACTTAGTTAAAACAGAATTTACCAATGAAACTTTAGATAAAATA
LysAspSerIleAsnAsnLeuValLysThrGluPheThrAsnGluThrLeuAspLysIle

      790       800       810       820       830       840
CAGCAGACACAAGACTTATTAAAAAAGATACCTAAGGATGTACTTGAAATTTATAGTGAA
GlnGlnThrGlnAspLeuLeuLysLysIleProLysAspValLeuGluIleTyrSerGlu

      850       860       870       880       890       900
TTAGGAGGAGAAATCTATTTTACAGATATAGATTTAGTAGAACATAAGGAGTTACAAGAT
LeuGlyGlyGluIleTyrPheThrAspIleAspLeuValGluHisLysGluLeuGlnAsp

      910       920       930       940       950       960
TTAAGTGAAGAAGAGAAAAATAGTATGAATAGTAGAGGTGAAAAAGTTCCGTTTGCATCC
LeuSerGluGluGluLysAsnSerMetAsnSerArgGlyGluLysValProPheAlaSer

      970       980       990      1000      1010      1020
CGTTTTGTATTTGAAAAGAAAAGGGAAACACCTAAATTAATTATAAATATCAAAGATTAT
ArgPheValPheGluLysLysArgGluThrProLysLeuIleIleAsnIleLysAspTyr

     1030      1040      1050      1060      1070      1080
GCAATTAATAGTGAACAAAGTAAAGAAGTATATTATGAAATTGGAAAGGGGATTTCTCTT
AlaIleAsnSerGluGlnSerLysGluValTyrTyrGluIleGlyLysGlyIleSerLeu

     1090      1100      1110      1120      1130      1140
GATATTATAAAGTAAGGATAAATCTCTAGATCCAGAGTTTTTAAATTTAATTAAGAGTTTA
AspIleIleSerLysAspLysSerLeuAspProGluPheLeuAsnLeuIleLysSerLeu

     1150      1160      1170      1180      1190      1200
AGCGATGATAGTGATAGTAGCGACCTTTTATTTAGTCAAAAATTTAAAGAGAACCTAGAA
SerAspAspSerAspSerSerAspLeuLeuPheSerGlnLysPheLysGluLysLeuGlu

     1210      1220      1230      1240      1250      1260
TTGAATAATAAAAGTATAGATATAAATTTTATAAAAGAAAATTTAACTGAATTTCAGCAT
LeuAsnAsnLysSerIleAspIleAsnPheIleLysGluAsnLeuThrGluPheGlnHis

     1270      1280      1290      1300      1310      1320
GCGTTTTCTTTAGCGTTTTCTTATTATTTTGCACCTGACCATAGAACGGTATTAGAGTTA
AlaPheSerLeuAlaPheSerTyrTyrPheAlaProAspHisArgThrValLeuGluLeu

     1330      1340      1350      1360      1370      1380
TATGCCCCCGACATGTTTGAGTATATGAATAAGTTAGAAAAAGGGGGATTTGAGAAAATA
TyrAlaProAspMetPheGluTyrMetAsnLysLeuGluLysGlyGlyPheGluLysIle

     1390      1400      1410      1420      1430      1440
AGTGAAAGTTTGAAGAAAGAAGGTGTGGAAAAAGATAGGATTGATGTGCTGAAAGGAGAA
SerGluSerLeuLysLysGluGlyValGluLysAspArgIleAspValLeuLysGlyGlu

     1450      1460      1470      1480      1490      1500
AAAGCACTTAAAGCTTCAGGTTTAGTACCAGAACATGCAGATGCTTTTAAAAAAATTGCT
LysAlaLeuLysAlaSerGlyLeuValProGluHisAlaAspAlaPheLysLysIleAla
```

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      1510       1520       1530       1540       1550       1560
AGAGAATTAAATACATATATTCTTTTTAGGCCTGTTAATAAGTTAGCTACAAACCTTATT
ArgGluLeuAsnThrTyrIleLeuPheArgProValAsnLysLeuAlaThrAsnLeuIle

      1570       1580       1590       1600       1610       1620
AAAAGTGGTGTGGCTACAAAGGGATTGAATCAACATGGAAAGAGTTCGGATTGGGGCCCT
LysSerGlyValAlaThrLysGlyLeuAsnGluHisGlyLysSerSerAspTrpGlyPro

      1630       1640       1650       1660       1670       1680
GTAGCTGGATACATACCATTTGATCAAGATTTATCTAAGAAGCATGGTCAACAATTAGCT
ValAlaGlyTyrIleProPheAspGlnAspLeuSerLysLysHisGlyGlnGlnLeuAla

      1690       1700       1710       1720       1730       1740
GTCGAGAAAGGAAATTTAGAAAATAAAAAATCAATTACAGAGCATGAAGGTGAAATAGGT
ValGluLysGlyAsnLeuGluAsnLysLysSerIleThrGluHisGluGlyGluIleGly

      1750       1760       1770       1780       1790       1800
AAAATACCATTAAAGTTAGACCATTTAAGAATAGAAGAGTTAAAGGAAAATGGGATAATT
LysIleProLeuLysLeuAspHisLeuArgIleGluGluLeuLysGluAsnGlyIleIle

      1810       1820       1830       1840       1850       1860
TTGAAGGGTAAAAAAGAAATTGATAATGGTAAAAAATATTATTTGTTAGAATCGAATAAT
LeuLysGlyLysLysGluIleAspAsnGlyLysLysTyrTyrLeuLeuGluSerAsnAsn

      1870       1880       1890       1900       1910       1920
CAGGTATATGAATTTAGAATTAGCGATGAAAACAACGAAGTACAATACAAGACAAAAGAA
GlnValTyrGluPheArgIleSerAspGluAsnAsnGluValGlnTyrLysThrLysGlu

      1930       1940       1950       1960       1970       1980
GGTAAAATTACTGTTTTAGGGGAAAAATTCAATTGGAGAAATATAGAAGTGATGGCTAAA
GlyLysIleThrValLeuGlyGluLysPheAsnTrpArgAsnIleGluValMetAlaLys

      1990       2000       2010       2020       2030       2040
AATGTAGAAGGGGTCTTGAAGCCGTTAACAGCTGACTATGATTTATTTGCACTTGCCCCA
AsnValGluGlyValLeuLysProLeuThrAlaAspTyrAspLeuPheAlaLeuAlaPro

      2050       2060       2070       2080       2090       2100
AGTTTAACAGAAATAAAAAAACAAATACCCACAAAAAGAATGGATAAAGTAGTTAACACC
SerLeuThrGluIleLysLysGlnIleProThrLysArgMetAspLysValValAsnThr

      2110       2120       2130       2140       2150       2160
CCAAATTCATTAGAAAAGCAAAAAGGTGTTACTAATTTATTGATTAAATATGGAATTGAG
ProAsnSerLeuGluLysGlnLysGlyValThrAsnLeuLeuIleLysTyrGlyIleGlu

      2170       2180       2190       2200       2210       2220
AGGAAACCGGATTCAACTAAGGGAACTTTATCAAATTGGCAAAAACAAATGCTTGATCGT
ArgLysProAspSerThrLysGlyThrLeuSerAsnTrpGlnLysGlnMetLeuAspArg

      2230       2240       2250       2260       2270       2280
TTGAATGAAGCAGTCAAATATACAGGATATACAGGGGGGGATGTGGTTAACCATGGCACA
LeuAsnGluAlaValLysTyrThrGlyTyrThrGlyGlyAspValValAsnHisGlyThr
```

```
      2290       2300       2310       2320       2330       2340
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GluGlnAspAsnGluGluPheProGluLysAspAsnGluIlePheIleIleAsnProGlu

      2350       2360       2370       2380       2390       2400
GGTGAATTTATATTAACTAAAAATTGGGAGATGACAGGTAGATTTATAGAAAAAAACATT
GlyGluPheIleLeuThrLysAsnTrpGluMetThrGlyArgPheIleGluLysAsnIle

      2410       2420       2430       2440       2450       2460
ACGGGAAAAGATTATTTATATTATTTTAACCGTTCTTATAATAAAATAGCTCCTGGTAAT
ThrGlyLysAspTyrLeuTyrTyrPheAsnArgSerTyrAsnLysIleAlaProGlyAsn

      2470       2480       2490       2500       2510       2520
AAAGCTTATATTGAGTGGACTGATCCGATTACAAAAGCCAAAATAAATACCATCCCTACG
LysAlaTyrIleGluTrpThrAspProIleThrLysAlaLysIleAsnThrIleProThr

      2530       2540       2550       2560       2570       2580
TCAGCAGAGTTTATAAAAAACTTATCCAGTATCAGAAGATCTTCAAATGTAGGAGTTTAT
SerAlaGluPheIleLysAsnLeuSerSerIleArgArgSerSerAsnValGlyValTyr

      2590       2600       2610       2620       2630       2640
AAAGATAGTGGCGACAAAGACGAATTTGCAAAAAAAGAAAGCGTGAAAAAAATTGCAGGA
LysAspSerGlyAspLysAspGluPheAlaLysLysGluSerValLysLysIleAlaGly

      2650       2660       2670       2680       2690       2700
TATTTGTCAGACTATTACAATTCAGCAAATCATATTTTTTCTCAGGAAAAAAAGCGTAAA
TyrLeuSerAspTyrTyrAsnSerAlaAsnHisIlePheSerGlnGluLysLysArgLys

      2710       2720       2730       2740       2750       2760
ATATCAATATTTCGTGGAATCCAAGCCTATAATGAAATTGAAAATGTTCTAAAATCTAAA
IleSerIlePheArgGlyIleGlnAlaTyrAsnGluIleGluAsnValLeuLysSerLys

      2770       2780       2790       2800       2810       2820
CAAATAGCACCAGAATACAAAAATTATTTTCAATATTTAAAGGAAAGGATTACCAATCAA
GlnIleAlaProGluTyrLysAsnTyrPheGlnTyrLeuLysGluArgIleThrAsnGln

      2830       2840       2850       2860       2870       2880
GTTCAATTGCTTCTAACACATCAAAAATCTAATATTGAATTTAAATTATTGTATAAACAA
ValGlnLeuLeuLeuThrHisGlnLysSerAsnIleGluPheLysLeuLeuTyrLysGln

      2890       2900       2910       2920       2930       2940
TTAAACTTTACAGAAAATGAAACGGATAATTTTGAGGTCTTCCAAAAAATTATTGATGAA
LeuAsnPheThrGluAsnGluThrAspAsnPheGluValPheGlnLysIleIleAspGlu

      2950       2960       2970       2980       2990       3000
AAATAAATATATATAATTGTTTTTCTGAAAATTCATCATTTTAAAGAAGACACTAGGAAT
Lys

      3010       3020       3030       3040       3050       3060
TAAATAGATGTATTGAATAGTTATAGTAATGGTCTTGTATGGACATACCGCTTATACTTT
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